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FOREWORD

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Paula H. Stern 10/20/97
PI - Signature Date

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INTRODUCTION

The subject of the studies is the effects of thyroid hormone (T3) on bone. The purpose of the studies was to determine the role and pathways of local cytokine and growth factor production in the osteoporosis elicited by thyroid hormone. Published reports from our laboratory and elsewhere have shown that T3 has a dose-dependent biphasic effect on the production of insulin-like growth factor-I (IGF-I) in bone and osteoblastic cells, stimulating at low concentrations and inhibiting at higher concentrations. Also, T3 potentiated the effect of interleukin-1 (IL-1) to stimulate the secretion of the osteoclastogenic factor interleukin-6 (IL-6) by bone. The scope of the research for the first year was 1) to optimize conditions for T3 stimulation of IGF-I and IL-6 secretion in osteoblastic cells and to initiate studies on the mechanisms of these effects (Specific Aims IA and IIA) and 2) to initiate studies on the effects of IGF-I and IL-6 on the anabolic and resorptive responses, which would include identifying the most useful models, establishing the methods and obtaining the necessary reagents, including antisense oligonucleotides (Specific Aims IB and IIB).

The background for the studies is the recognition the thyroid hormone (T3) is a critical regulator of skeletal development and function, but that the mechanisms of the effects are poorly understood. The importance of T3 for normal bone development is apparent from the delayed skeletal maturation and abnormal bone characteristics in juvenile hypothyroidism and with mutations of the T3 receptor gene (Refetoff et al. 1993). Excessive T3 is a risk factor for osteoporosis (Riggs and Melton 1986, Baran and Braverman 1991), and postmenopausal women are particularly at risk (Guo et al. 1997). Histomorphometric studies show increases in both osteoblast and osteoclast activity in hyperthyroidism, with the changes being most marked in cortical bone (Bordier et al. 1967, Mosekilde and Melsen 1978). The net effect is a loss of bone mass. Excessive T3 for replacement or suppression is associated with decreased bone density (Franklyn and Sheppard 1990, Stoll et al. 1990, Diamond et al. 1991). Clinically, osteopenia induced by hyperthyroidism in young women with Graves' disease and in post-menopausal women over-treated with levothyroxin is a major contributor to osteoporosis.

Despite the clinical importance of the effects of T3 on bone, very little is known about the process and molecular mechanism by which this occurs. T3 stimulates bone resorption *in vivo* and *in vitro*. Excretion of pyridinium cross-links is elevated in hyperthyroidism and in patients on thyroid replacement therapy (Ernst and Froesch 1987). Histomorphometric analyses show increased osteoclast numbers and increases in resorbing surfaces, with loss of trabecular bone volume (Mosekilde and Melsen 1978). T3 stimulates cell replication in both rodent and human osteoblastic cells (Ernst and Froesch 1987, Kassem et al. 1993). At high, supraphysiological, concentrations, T3 inhibits replication (Sato et al. 1987, Kasono et al. 1988, LeBron et al. 1989, Egrise et al. 1990). T3 stimulates synthesis of type I collagen in bone organ cultures (Kawaguchi et al., 1994). The phenotypic marker of osteoblast activity, alkaline phosphatase, is increased by T3 in primary osteoblasts (Egrise et al. 1990), and in ROS17/2.8 (Sato et al. 1988) and MC3T3-E1 cells (Kasono et al. 1988). Alkaline phosphatase is also elevated in patients with hyperthyroidism (Cooper et al. 1979). Likewise, osteocalcin is increased by T3 in ROS17/2.8 cells (Sato et al. 1987), and is elevated in patients with hyperthyroidism (Lee et al. 1990). Thus, T3 stimulates both the breakdown of bone and the synthesis of new bone. For T3 to produce net bone loss, there must be a disproportionate or uncompensated increase in the resorptive actions when bone is exposed to supraphysiological concentrations of T3. Determining the mechanism by which this occurs is the goal of this research.

The osteoblast appears to be the critical target cell for T3 action in bone since T3 fails to activate isolated osteoclasts to resorb in the absence of osteoblasts (Allain et al. 1992). Receptors for T3 were demonstrated by binding studies in rodent and murine osteoblast cell lines and normal osteoblasts (Sato et al. 1987, Kasono et al. 1988, LeBron et al. 1989, Egrise et al. 1990, Rizzoli et al. 1986, Krieger et al. 1988). The

osteoblastic cell lines UMR-106 and ROS17/2.8 express both T3R 1 and T3R 1 as well as RXR and RAR isoforms (Williams et al. 1994). Transient gene expression assays and treatment of cells with T3, calcitriol and 9-cis-retinoic acid provide evidence that receptor complexes are functional, and even enhancing, in these cells. In recent years it has become apparent that osteoblasts are sites for the synthesis of local growth factors and cytokines, and these substances may mediate the effects of systemic hormones. Two local factors that are particularly likely to be involved in the actions of T3 on bone are insulin-like growth factor-I (IGF-I) and interleukin-6 (IL-6). IGF-I has significant anabolic effects on bone, increasing cell replication and both collagen and non-collagen protein synthesis (Canalis 1980, Hock et al. 1986, McCarthy et al. 1989, Centrella, et al. 1990, Pirskanen et al. 1993). Schmid, et al. reported stimulation of IGF-I by T3 in normal rat calvarial osteoblasts (Schmid et al. 1992). Our own studies demonstrate a dose-dependent, biphasic stimulation of IGF-I secretion in UMR-106 cells and fetal rat bone organ cultures (Lakatos et al. 1993). Klaushofer and colleagues (Klaushofer et al. 1994, Klaushofer et al. 1995) found an increase in IGF-I mRNA in MC3T3-E1 cells treated with T3. IL-6, a potent multifunctional cytokine, is a potent stimulator of osteoclast differentiation from precursor cells in human marrow (Kurihara et al. 1990). Hypercalcemia is elicited in nude mice injected with by Chinese hamster ovary cells that had been transfected with the murine interleukin-6 gene (Black et al. 1991). IL-6 is present in high concentrations in patients with Paget's disease of bone (Roodman et al. 1992). Resorption of dentin *in vitro* by giant cells from human giant cell tumors of bone was inhibited by antisense oligonucleotides to IL-6 (Reddy et al. 1994), and both IL-6 and IL-6R mRNA are higher in Pagetic osteoblasts and osteoclasts than those from normal remodelling bone (Hoyland et al. 1994). Other pathologic states in which IL-6 appears to have a role in bone resorption include multiple myeloma, rheumatoid arthritis and Gorham-Stout disease (Manolagas and Jilka 1995). We have found that T3 potentiates the IL-1-stimulated production IL-6 in bone (Tarjan and Stern 1995). In collaborative studies with Dr. Peter Lakatos at Semmelweis University Medical School in Budapest, Hungary, we have also found that circulating IL-6 is higher in hyperthyroid (Graves disease and toxic nodular goiter) than in euthyroid premenopausal women (Lakatos et al. 1997). Monocytes from the patients with Graves disease or toxic nodular goiter had elevated IL-6 production compared with controls (Lakatos et al. 1997). Other studies have shown the potentiation by T3 of IL-1-stimulated IL-6 production in human osteosarcoma cells (MG-63) and also noted increases in IL-6 and IL-6 soluble receptor in hyperthyroid patients (Passeri et al. 1995).

Thus, the well-established importance of T3 in the normal development of the skeleton as well as the potential increased fracture risk with thyroid excess make it essential that we understand how T3 acts on bone. At the current time there is virtually no molecular information on how T3 influences bone physiology. The studies we have undertaken are designed to elucidate the molecular mechanisms by which T3 affects the production of IGF-I and IL-6 in bone and to address the critical question of whether T3-stimulated increases in these factors is essential for the actions of T3 in bone.

The specific aims of the project were as follows:

- IA: Determine the mechanism of the effect of T3 to increase IGF-I in osteoblastic cells
- IB: Determine the biological significance of the increased IGF-I for the anabolic effects of T3
- IIA: Determine the mechanism and modulation of T3 potentiation of IL-6 production
- IIB: Determine the biological significance of the T3 potentiation of IL-6 production

The studies have been carried out as a coordinated effort between the laboratories of Drs. Stern and Madison. For the purpose of the report, we have separated the presentation of the results and discussion of the work carried out in Dr. Madison's laboratory (Specific Aims IA and IIA) and those carried out in Dr. Stern's laboratory (Specific Aims IB and IIB).

BODY OF THE REPORT

Experimental Methods

Cells: For the studies carried out during the first year, we used several osteoblastic cell models. These included UMR-106 cells, a rat osteoblastic cell model that we had used for our studies on IGF-I secretion (Lakatos et al. 1993), MC3T3-E1 cells, a mouse immortalized cell line, which has been used by other investigators for studies of thyroid hormone action (Varga et al. 1994, Klaushofer et al 1995), ROS 17/2.8 cells, a rat osteoblastic cell line that is more differentiated than the UMR-106 cells, normal neonatal mouse calvarial cells, and Saos2, MG-63, HOS, G292 Fob and Hob human osteoblastic cells.

Culture Media and Hormone Treatments: Cells were cultured in the recommended media in the presence of 5% fetal calf serum (FCS). For experiments involving hormonal manipulation, cells were pre-cultured in hormone depleted media for 12-18 hours, prior to IL-1 and/or T3 treatment. Two methods were assessed for hormone depletion; charcoal stripping, which removes to near completion all steroid and peptide hormones, and Dowex AF-1-X-10 resin stripping (Samuels et al 1979), which removes T3 and T4 hormones with reasonable selectivity. Charcoal stripping leaves a profoundly nutritionally deprived media and essentially arrests cell division. Resin-treated serum is better at supporting cell growth, but the relative concentrations of other relevant hormones (retinoids, Vitamin D, interleukins) is unknown. In general, resin-treated serum gave the best results, allowing better cell growth and greater IGF-I and Il-6 production and a larger T3 effect.

Organ cultures: For the studies carried out during the first year, we used fetal rat limb bone cultures, in which the effects of T3 to stimulate resorption are direct, and not mediated through prostaglandin production (Mundy et al. 1979, Hoffmann et al. 1986, Kawaguchi et al. 1994).

Time course: Incubations were maintained from 1-5 days, depending upon the experiment. Cell were used at confluency, except for studies on cell replication. UMR-106 cells were from passages 5-20. MC3T3-E1 cells were maintained in culture with -MEM + 5% fetal bovine serum, 50 µg/ml ascorbic acid and 50 µg/ml gentamicin for 18-21 days, to allow differentiation to the osteoblast phenotype before they were used.

Thymidine incorporation: Cells are plated in DMEM or µ-MEM + 5% heat-inactivated fetal bovine serum (FBS) T3/T4-free serum at concentrations that would permit achievement of confluency in 96 hours (35,000 cells/well in a multiwell dish). After 48 hr (at which the cells are at 70% confluency) the medium is changed to 0.1% bovine serum albumin, for an overnight incubation to quiesce the cells. They are then treated with T3 and harvested at 24 hr. Two hours before the end of the incubation, 0.5 µCi/ml ³H- thymidine is added. Incubations are in 5% CO₂ at 37°C. The cells are then washed 2x with 500 µl cold phosphate-buffered saline (PBS) and reaction is stopped with cold 10% TCA. The TCA is removed and the cells harvested with 750 µl 0.5 N NaOH and 0.1% sodium dodecyl sulfate (SDS). An 100 µl aliquot is used for liquid scintillation counting.

Proline incorporation: Collagen synthesis is estimated by incorporation of ³H-proline into collagenase-digestible protein. Cells are plated and treatments added after 24 hr. Tissues are incubated for 2 x 72 hr with the treatments. For the final 2 hr, 5 µCi/ml ³H-proline is added. The reaction is stopped with 10% TCA, the cells washed, and the incorporation into the TCA-precipitable fraction measured by liquid scintillation counting.

Alkaline phosphatase: Cells are used at confluency in 96 well plates, plated at 16,000 cells/well, except for MC3T3, which are maintained in culture (D-MEM + 5% resin-stripped FBS, 50 µg/ml ascorbic acid, 50 µg/ml gentamicin for 21 -days). Alkaline phosphatase in cell lysates or culture media was measured by the

production of p-nitrophenol from p-nitrophenyl phosphate (Schlossman, et al. 1982). Cells incubated with the selected treatments for 72 hr. Cells are washed with cold PBS. The medium is aspirated, 100 μ l diethanolamine, 50 mM, pH 10.5 is added, and μ l of 2.5 mM p-nitrophenylphosphate in glycine buffer is added. Medium or tissue extracts are incubated for 30 min with the substrate at pH 10.5 (100 mM glycine/2mM $MgCl_2$ buffer) and the reaction stopped by the addition of 0.1 N NaOH. Absorbance is read on a Varian spectrophotometer at 410 nm and activity calculated in reference to a standard curve of p-nitrophenol.

Osteocalcin: Osteocalcin secreted by the tissues after 72 hr from the same cultures used for alkaline phosphatase is measured by radioimmunoassay according to procedures provided by Biomedical Technologies. A radioimmunoassay using a goat antiserum to mouse osteocalcin and mouse [^{125}I]- osteocalcin tracer (Biomedical Technologies, Stoughton, MA) was used. This assay recognizes total osteocalcin.

Receptor Binding: IGF-I receptor binding was measured in membrane fractions using ^{125}I -IGF-I (Amersham). Cells grown in 24-well dishes were treated in triplicate. At the end of the incubation, they were washed with 1 ml 222 mM sucrose in NaCl-free Hank's solution containing 0.5% BSA, 20 mM HEPES, pH 7.4. For nonspecific binding, 300 nM IGF-I was added to each well. 20,000 cpm ^{125}I -IGF-I was added. The cells were incubated for 2 hr. Supernatant was decanted, the cells washed 2X with the above buffer, solubilized with 0.5 ml 1 N NaOH, transferred to counting tube followed by another 0.5 ml wash of the well. Cells were centrifuged 2000 x g for 5 minutes and the precipitate counted in a counter.

Statistics: Except where otherwise indicated, experiments were done in triplicate or higher. Data were analyzed by Analysis of Variance with the Newman-Keuls post-test. For all figures, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ vs. control; # = $p < 0.05$, ## = $p < 0.01$, ### = $p < 0.001$ vs. treatment (IGF or T3).

Western Immunoblotting: IGF-I receptor expression was determined in whole cell lysates of MC3T3-E1 cells by methods described by us previously (Sanders and Stern 1996). The IGF-I receptor antibody was from Santa Cruz.

RT-PCR Assays: The RT-PCR measurements in these experiments were performed as non-competitive reactions which compare the relative expression of an experimental gene to an appropriate control, housekeeping gene (cyclophilin, GAPDH, 18S rRNA). All RT-PCR measurements are performed in the linear range of amplification and the assay is performed in such a manner that the linearity is confirmed before the data is considered valid. Equal aliquots of total RNA are converted to cDNA with reverse transcriptase. Aliquots of the cDNA are subjected to PCR using Taq polymerase, dNTP, Mg^{++} , and 20 pmole of oligonucleotide primer pairs. PCR products are detected after electrophoresis by phosphorimager quantification of ^{32}P -dNTP incorporated into the product during PCR. To assure a linear assay a set of serial dilutions ($n=3-5$) of the cDNA template (100-500 ng total) is made for each individual sample, spanning 3-4 logs of concentrations. The yield of product from the experimental gene PCR is compared to that of a housekeeping gene for each of the cDNA dilutions (usually cyclophilin). If PCR is occurring in a linear fashion, the ratios of these comparisons remains constant across a dilution series of the sample. The ratios obtained from linear regions of the dilution series are used to determine a Mean and Std. Error for the relative relationship between the experimental gene and the control gene. Comparison can then be made between these relative ratios in different experimental situations.

Transient Gene Expression and Luciferase Assays: For transfections studies, cells were plated in 24 well plates, transferred to resin-stripped serum containing media for 18-24 hours. The osteosarcoma cells were found to be very difficult to transfect, and generated only weak luciferase signals in cellular extracts following transient gene expression. The following DNA transfections methods were assessed; calcium phosphate, DEAE Dextran, electroporation, DOTAP liposomes (Boehringer), soybean lipids with dimethyldioctadecylammonium bromide (DDAB) liposomes, and Transfect reagent (Promega). No method

generated even moderate amounts of luciferase signal using two panels of experimental promoters and strong viral promoters as controls. Simultaneous transfection of other cell lines which are easier to transfect (Jeg-3, HeLa) demonstrated that the technique of transfection and luciferase assay was working. This suggests that the problem may not be with the transfection techniques themselves, but that the osteosarcoma cells only weakly express the luciferase cDNA under any condition. A review of the available literature using osteosarcoma cells suggests that this is a common technical obstacle in performing transient gene expression in this type of cell. The most consistently successful methods were electroporation and soybean lipid liposomes with DDAB (Rose et al 1991). The following promoter constructs were examined:

IGF-I	-2.0 Kb of 5' flank of IGF-I in pGL2 Basic (Lowe et al 1992)
	-846 bp of 5' flank of IGF-I in pGL2 Basic
	-197 bp of 5' flank of IGF-I in pGL2 Basic
IL-6	-742 bp of Il-6 promoter in pGL2 Basic (gift of Dr. Bernard Stern) (Stern et al 1995)
	-224 bp of Il-6 promoter in pGL2 Basic
	-158 bp of Il-6 promoter in pGL2 Basic
	-109 bp of Il-6 promoter in pGL2 Basic
	-49 bp of Il-6 promoter in pGL2 Basic
Control Promoters	
	pGL2 Basic
	SV40 promoter in pGL3 Basic
	RSV400 in PA3Luc

Luciferase activity in total cellular extracts of cells transfected for 24 to 48 hours was measured for 10 seconds in a luminometer by standard methods.

Materials: T3, All-trans retinoic acid and IGFBP-3 were from Sigma, Vitamin D and 9-cis retinoic acid, Hoffman-LaRoche, IGF-I from Gibco or Biosource, Interleukin-1 Biosource, PTH, NovaBiochem, IL-1 and IL-6 antibodies, Endogenan, antibodies to IL-6 and IL-6 receptor from Biosource, and IGF-IR-Ab was from Calbiochem. Antisense oligonucleotides to the rat/mouse IGF receptor were synthesized by the Biotechnology Center at Northwestern University.

Assumptions

The models, materials and design used were based on several assumptions, with some testing of these incorporated into the design:

- 1) It was assumed that the models were representative of the osteoblast phenotype. However, we also compared several of the responses in the different models to determine whether there were any important difference in the responses of the different models to T3 and to IGF-I. This could help us to assess how generally we could extrapolate our findings.
- 2) It was assumed that the parameters we measured would reflect anabolic or catabolic responses. In the case of the anabolic responses, we measured several different parameters. In the case of the catabolic, it was assumed that ^{45}Ca release was a valid measure of resorption. This is supported by correlations between changes in ^{45}Ca release and loss of bone mass, as well as blinded visual scoring of the degree of resorption.

Procedures

Covered under Experimental Methods

Results and Discussion. Specific Aims IA and IIA (Dr. Madison)

Classification of Experimental Techniques and Subject

1. T3 binding assays with nuclear extracts from osteosarcoma cells
2. Identification of thyroid receptor (TR) isoforms in osteosarcoma cells by Western blotting with isoform specific anti-TR antibodies
3. T3 effects on IGF-I mRNA production in human and rodent osteosarcoma cell measured by RT-PCR
4. T3 effect on IL-6 protein production measured by ELISA
5. T3 effect on IL-6 mRNA production measured by RT-PCR
6. T3 effect on IL-1 receptor production measured by RT-PCR
7. Transient gene expression analysis of IGF-I and IL-6 promoter regulation by T3

1. T3 binding assays with nuclear extracts from osteosarcoma cells: Nuclear extracts were prepared from large scale culture of HOS, and MG-63 cells. Positive control extracts were prepared from rat pituitary GH4 cell which have abundant thyroid receptors. C6 glioma cell nuclear extracts were prepared as a negative control. Protein content of the extracts was measured by a Coomassie blue binding assay. Equal amounts of protein, 5 ug, were incubated with 1.0 nM ^{125}I -T3 (>2000 Ci/mmol) in the presence or absence of 1 mM non-radioactive T3. Specific binding was observed in the osteosarcoma cell nuclear extracts and in the GH4 cell line, while negligible binding was present in the C6 extracts (*Figure A1*). Additional binding studies using a cold competition technique (not shown) demonstrated by Scatchard analysis that the binding was of high affinity, ($K_D \sim 0.1$ nM), comparable to that in GH4 extracts.

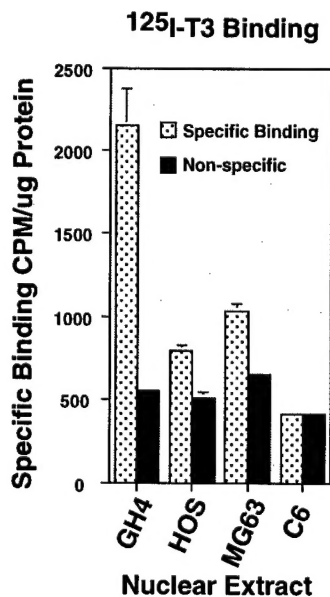


Figure A1 Demonstration of specific binding of ^{125}I -T3 to nuclear extracts prepared from osteosarcoma cells (HOS and MG-63).

2. Identification of thyroid receptor (TR) isoforms in osteosarcoma cells by Western blotting with isoform specific anti-TR antibodies: In order to document that the high affinity binding was due to the presence of TR, the TR isoforms were identified by Western blotting. Anti-receptor antibodies had been prepared previously by Dr. Madison by immunization of rabbits with synthetic isoform specific peptides or whole recombinant TR protein produced in *E. coli*. 10 μ g of nuclear extract was separated by electrophoresis with 10% SDS-PAGE and transferred to nitrocellulose in Towbin's buffer. The anti-TR α 1 antibody reacted strongly with a major band in GH4, HOS, and MG-63 cells and with purified TR α 1 but not with purified TR β 1 (*Figure A2*). The second antibody is broadly specific and interacts with both TR isoforms. Bands were identified in GH4 cells, HOS, MG-63 and with both purified TRs. The major band labeled with the broadly specific antibody co-migrated with the *E. coli* produced TR β 1, suggesting it was the major isoform seen in the osteosarcoma cell extracts.



Figure A2 Western blot analysis of GH4, osteosarcoma cell extracts, and purified TR isoforms.

3. T3 effects on IGF-I mRNA production in human and rodent osteosarcoma cell measured by RT-PCR. RT-PCR was performed 2 - 3 times with each of five different human osteosarcoma cell RNA extracts and IGF-I cDNA was detected only in SaoS-2 cells (*Table I*). In SaoS-2 cells the level of expression was very low, making the detection and comparison with the biological control (cyclophilin) impossible because of non-linear amplification and large difference in cycle number required for amplification. Shown in *Figure A3* is the result of a less optimal alternative analysis representative of one of four similar experiments done with these cells. The amount of radioactivity (CPM) in the amplified band per amount of cDNA template subjected to PCR is plotted against the concentration of T3. The cells were treated with a range of concentrations of T3 (0 to 10^{-6} M) for 48 hours in this experiment. Other experiments examined longer and shorter exposure time, 12 hours to 96 hours of T3 treatment (not shown). The data suggest a positive linear relationship between T3 dose and IGF-I expression. However, the level of expression of the cDNA is so low that firm conclusions should not be drawn from the data. Larger amounts of easily measurable IGF-I message was detected in Ros 17/2.8 cells. However, there was an unusual cross interference between the amplification of the cyclophilin control PCR product and the amplified IGF-I band making the data unusable. In the Ros 17/2.8 experiments there was an observable trend, despite the artifacts, toward increased IGF-I in the presence of higher doses of T3. We expect this problem to be resolved using the 18S Competicon primers as controls when the experiments are repeated.

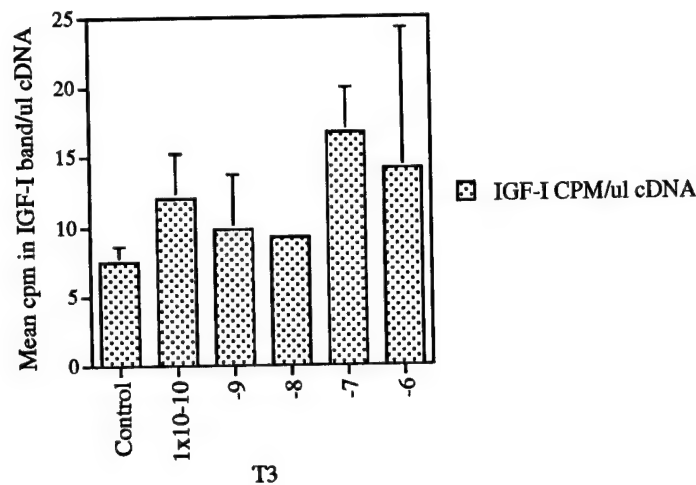


Figure A3 RT-PCR of IGF-I RNA from SaoS-2 cells treated with a range of concentrations of T3. The less optimal method of directly comparing the incorporated radioactivity in the amplified band between different experiments was used in this experiment because of extreme differences in the expression level of the IGF-I mRNA and all of our control genes (cyclophilin, GAPDH, actin)

4. T3 effect on IL-1 induced IL-6 production measured by ELISA. The influence of T3 on IL-1 induced IL-6 secretion was extensively studied in over 50 experiments using six human osteosarcoma cell lines. IL-6 was measured by ELISA using the manufacturers instructions and recommendations (Endogen). IL-6 standard curves were developed using IL-6 supplied by the manufacturer. Variables of cell density, time in culture, passage number, time of pre-treatment hormone depletion, time of T3 treatment, time of IL-1 treatment, dose of IL-1 and dose of T3 were examined in the two major cell types studied, SaoS-2 and MG-63. Impressive synergistic T3 effects were often observed as in *Figure A4a* which shows the secreted IL-6 (pg/ml) from SaoS-2 cells, pre-treated with T3 10⁻⁶ M for 72 hours prior to IL-1 treatment (0.05 ng/ml) for another 8 hours in the continued presence of T3. *Figure A4b* shows a more detailed analysis of the early time course of IL-1 treatment as before in the presence or absence of T3. The largest effects on IL-6 induction caused by T3 were seen at times beyond 8 hours.

However, the synergy between IL-1 and T3 was not always observed. An example showing little or no T3 effect on secreted IL-6 from HOS and MG-63 cells is shown in *Fig, A4c*. It is clear however from these experiments that T3 itself does not directly induce IL-6 secretion by itself from these cells. An example of both T3 and IL-1 dose response curve, in MG-63 cells, with T3 (various doses) treatment for 72 hours prior to an additional 72 hrs of IL-1 (range of doses) treatment in the presence of T3 is shown in *Figure A4d*. From this and other experiments using SaoS-2 and MG-63 cells primarily, but also the other human cell lines shown in *Table I*, it was determined that the optimal synergy of action between IL-1 and T3 for IL-6 production was accomplished with 1-3 days of pre-treatment of cells with high dose T3 (10⁻⁶ M) followed by low to moderate dose (0.05 ng/ml) of IL-1 for an excess of 24 hours. These conditions most often produced evidence T3/IL-1 synergy, however it should be noted that in a number of well performed experiments under what we considered optimized conditions there was no or little evidence of any interaction between the biology of T3 and IL-1. As best we could determine this was an effect intrinsic to the cell culture itself and likely included factors of cell passage number, cell density, time since plating, and frequency of medium changes during hormonal treatments.

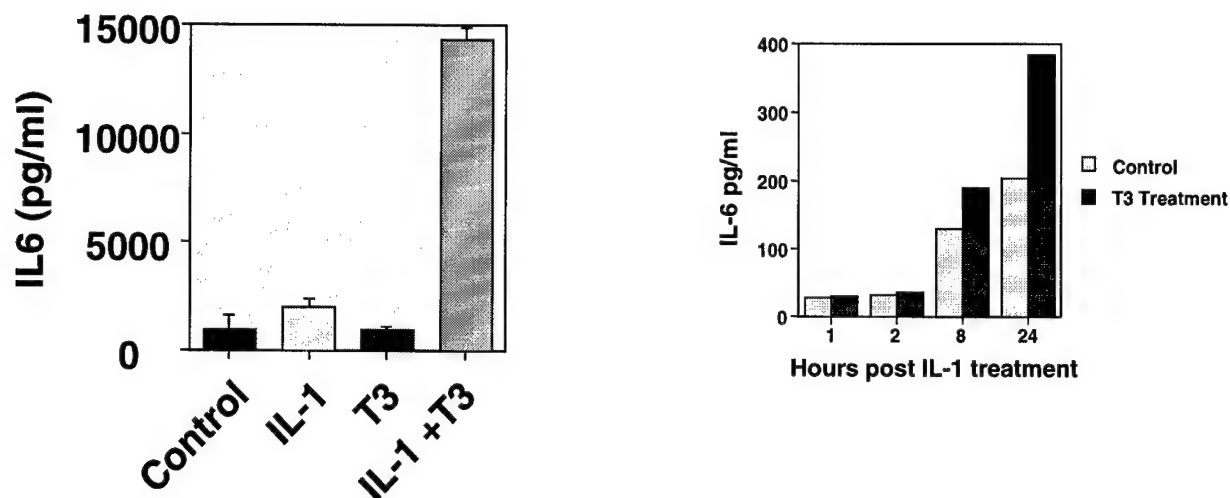


Figure A4a (left) Secreted IL-6 from SaoS-2 cells following treatment with and without T3 and IL-1

Figure 4Ab (right) Early time course of IL-6 secretion from SaoS-2 cells induced by IL-1 with and without pretreatment with T3

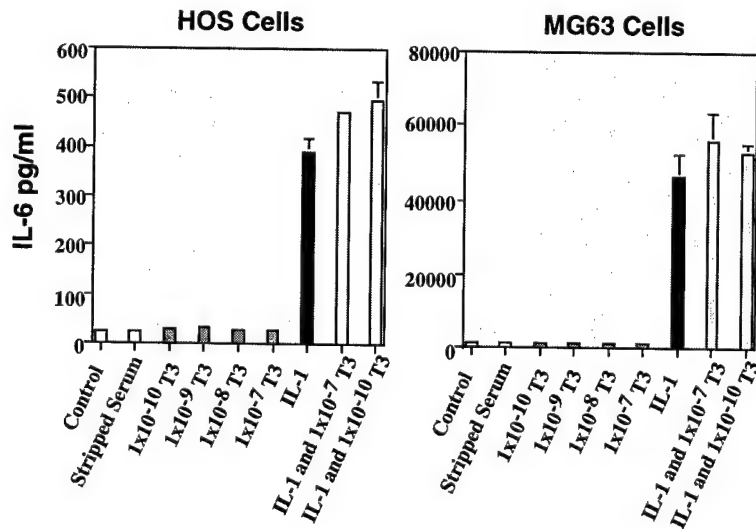


Figure A4c Unstimulated and IL-1 stimulated IL-6 secretion from HOS and MG-63 cells after treatment with a range of doses of T3

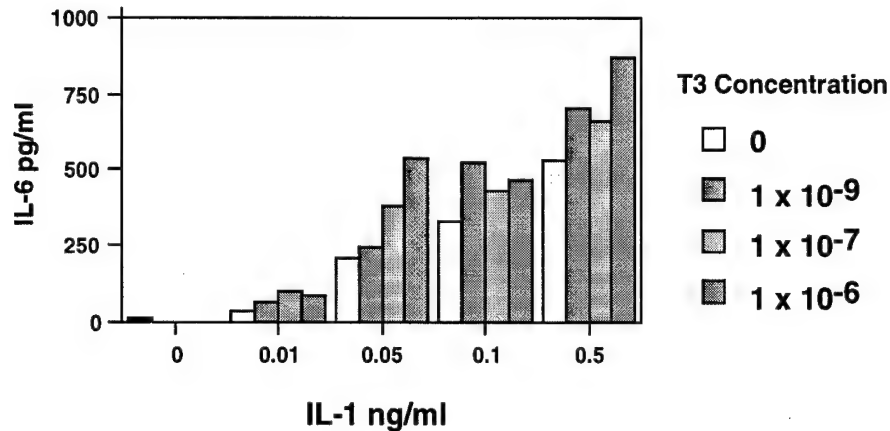


Figure A4d Dose response for T3 and IL-1 induced IL-6 secretion from MG-63 cells.

5. T3 effect on IL-6 mRNA production measured by RT-PCR: The production of IL-6 mRNA was examined in total RNA prepared from those experiments described above where a synergistic response to T3 and IL-1 was observed by ELISA measurement of secreted IL-6 protein.. More than 50 RT-PCR experiments were performed using the six human osteosarcoma cell lines. Data was obtained by comparing the ratios of radioactivity in the experimental and control bands as measured with a phosphorimager. These ratios were from a series of dilutions of cDNA which were amplified at the same time. In order to verify the linearity of the PCR, the ratios are plotted, and the slope of the resulting lines evaluated for slope and distribution. If the slope of the line was between -1.0 and +1.0 (i.e. it is relatively flat) and the R value for the equation of the line was greater than 0.7 the assay was considered valid. An example of a high quality assay is shown in *Figure A5a*. This experiment shows the measured amount of IL-6 cDNA with and without T3 and IL-1. The data points generating the lines as in *Figure A5a* were averaged and their distribution used to generate a Standard Error and the data represented as in *Figure A5b*. In this experiment there is an approximate doubling of the amount of IL-6 mRNA in the cells treated with IL-1 and T3. In agreement with the ELISA results, T3 did not stimulate IL-6 production by itself. The magnitude of the measured mRNA increase was generally lower than that observed with IL-6 secretion by ELISA. In this particular case the ELISA measurements had shown a 7 fold increase in IL-6 in the T3 + IL-1 treated cells. For completeness sake we also examined IL-6 mRNA levels in some experiments where there had been no observed synergy in T3 and IL-1 action. In these cases the measured IL-6 levels were invariably the same. The measurement of IL-6 mRNA from a variety of experiments led us to conclude that when culture conditions were such that a synergistic effect of T3 and IL-1 had occurred, there was often a 1.5 to 2.0 fold increase in the amount of IL-6 mRNA.

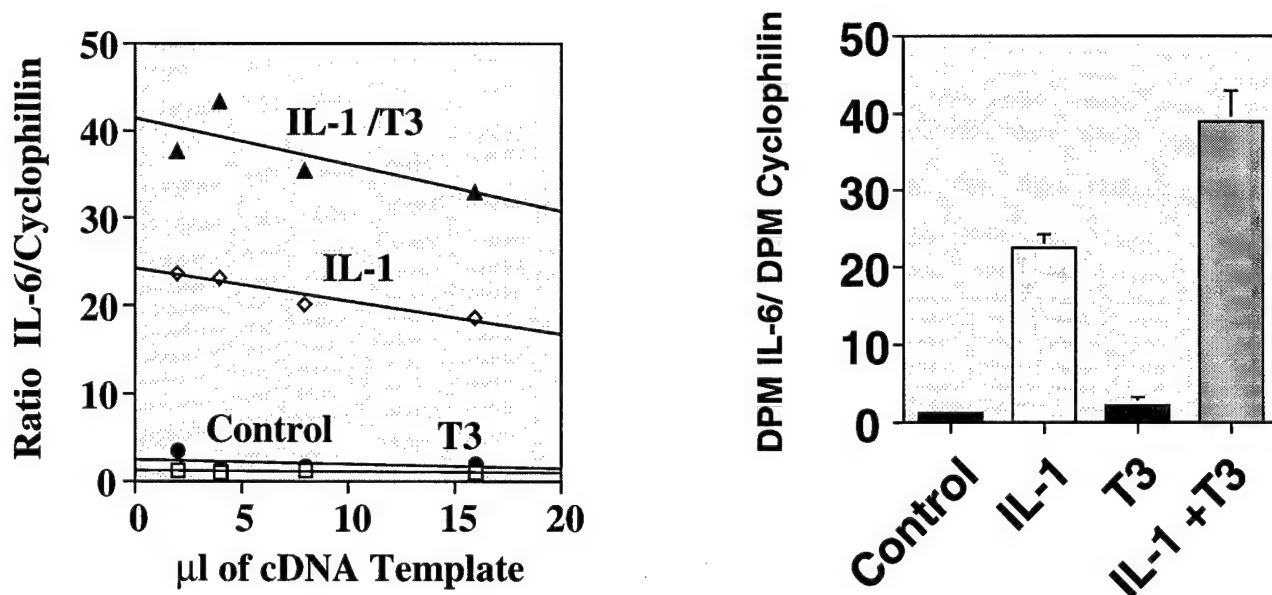


Figure A5a (left) RT-PCR measurement of IL-6 and cyclophilin mRNA levels in MG-63 cells following treatment with and without T3 and IL-1. Analysis of IL-6 to cyclophilin ratios after phosphor imager measurement of RT-PCR reactions.

Figure A5b (right) A transformation of the data in Figure A5b achieved by determining the Mean and Std. Error of the different ratio points.

6. T3 effect on IL-1 receptor production measured by RT-PCR: A previous study on Vitamin D action had suggested that Vitamin D potentiated the action of IL-1 on osteoblast production of IL-6 by increasing the expression of the IL-1 receptor (IL-1R) (Lacey et al 1991). Because of the evident parallelism of this to the T3 physiology being studied we sought to determine if this was occurring as part of the observed effect of T3. The mRNA for the IL-1 receptor was measured in 3 experiments in both MG-63 cells and SaoS-2 cells, comparing its expression to the level of cyclophilin after treatment with and without T3 and IL-1. (Figure A6). A representative results is shown indicating that we consistently observed a decrease of between 50 and 75% of the amount of IL-1R in T3 treated cells. The effect of T3 was thus unexpectedly the opposite of that observed with Vitamin D and changes in IL-1 receptor level were not likely a cause for T3 potentiation of IL-1 induced IL-6 production. Unlike the observed T3/IL-1 synergy to increase IL-6 levels, this effect appeared to be the result of direct independent action of T3 and was not qualitatively affected by co-treatment with IL-1.

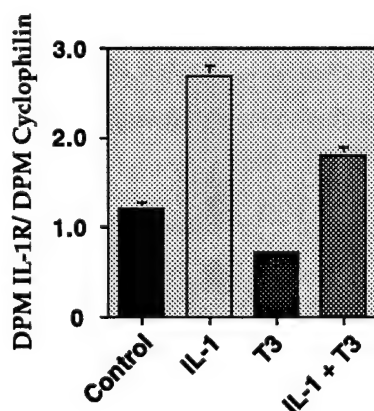


Figure A6 RT-PCR measurement of IL-1R expression in MG-63 cells following treatment with IL-1 and T3.

Results - Specific Aims IB and IIB - Dr. Stern

³H-Thymidine incorporation: These studies have been carried out in MC3T3 mouse osteoblastic cells, UMR-106 rat osteoblastic osteosarcoma cells and primary mouse osteoblasts. The rate of cell proliferation was highest in the UMR-106 cells and slowest in the normal osteoblasts. Incubation with IGF-I for 24-48 hr stimulated this parameter in all three cell types (*Figure B1a-c*). The effects of 10 nM IGF-I were inhibited in the presence of 1.5 µg/ml of an antibody to the IGF receptor (*Figure B2*). This same concentration of antibody failed to inhibit the effect of a higher concentration, 100 nM, of IGF-I (data not shown). In contrast to the effects seen with IGF-I, T3 failed to stimulate ³H-thymidine incorporation in any of the cell types (*Figure B3*).

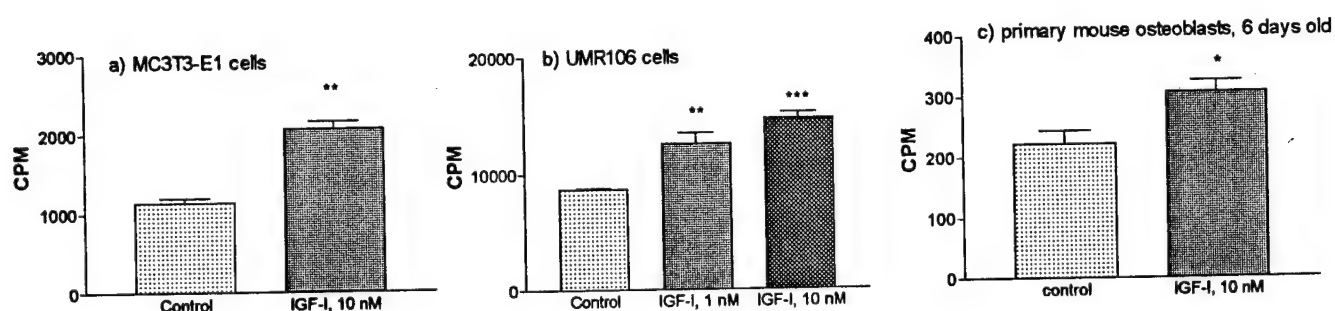


Figure B1. Effect IGF-I on thymidine incorporation in a) MC3T3-E1 osteoblastic cells, b) UMR-106 osteoblastic osteosarcoma cells, c) primary mouse osteoblasts. Values are cpm/well.

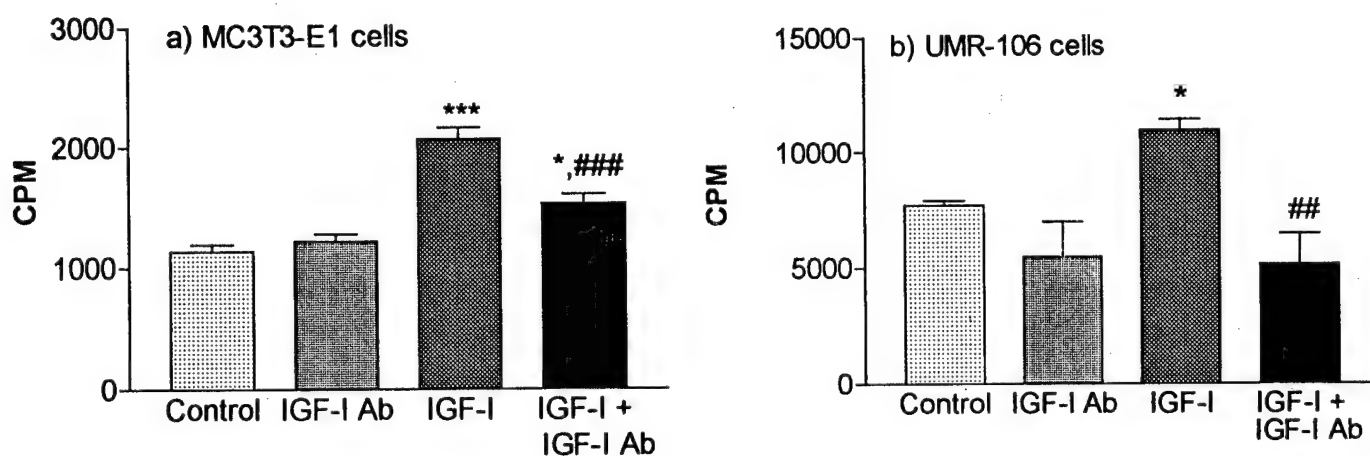


Figure B2. Inhibition of effects of 10 nM IGF-I on thymidine incorporation by antibody to IGF-I receptor (1.5 µg/ml) in a) MC3T3-E1 cells and b) UMR-106 cells.

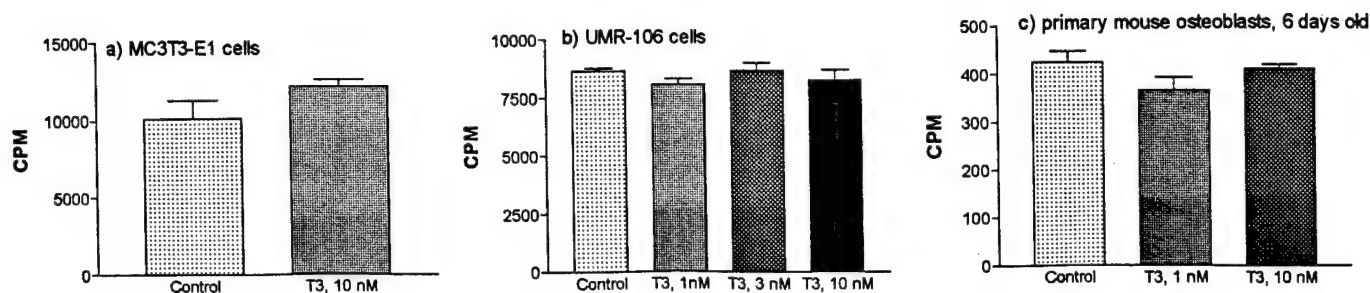


Figure B3. Lack of effect of T3 on thymidine incorporation in a) MC3T3-E1 cells, b) UMR 106 cells and c) primary mouse osteoblasts.

The conclusions from these experiments are that under the conditions used, T3 did not affect cell replication in three different cell lines, all of which showed proliferative responses to IGF-I. The study also showed the effectiveness of the IGF-I receptor antibody to block IGF-I effects.

Alkaline phosphatase activity: The studies on this marker of differentiated osteoblastic activity were initially carried out in MC3T3 mouse osteoblastic cells, UMR-106 cells and neonatal mouse calvarial cells. Of these, the MC3T3 cells were the most satisfactory. The MC3T3 cells generally gave good responses to IGF-I and T3 (*Figure B4*), with the best effects with T3 obtained with a 72 h treatment with 10 nM T3. The inactive T3 analog, diiodotyrosine (DIT), failed to increase the alkaline phosphatase (*Figure B4*). The UMR-106 cells had very high endogenous alkaline phosphatase activity, and even serum starving the cells failed to decrease this. No further stimulation was seen with T3 (*Figure B5a*) or IGF-I (data not shown). With the mouse calvarial cells, although small effects of T3 were detected in some experiments (*Figure B5b*), in other experiments we failed to observe any response. The model was deemed too insensitive to be used for studies on the effects of antagonists, especially as it would have required the use of large numbers of animals to obtain statistically significant results.

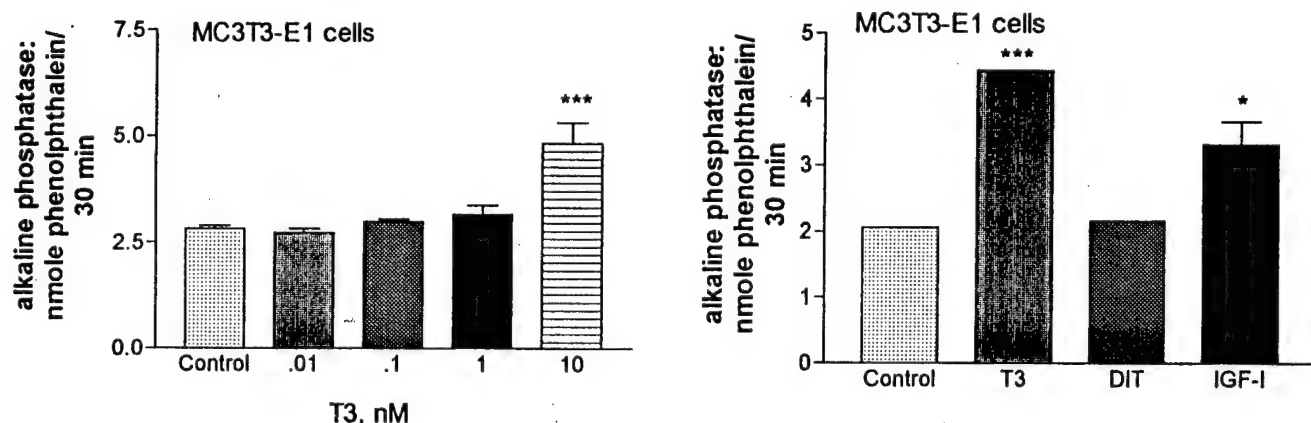


Figure B4. Effects of IGF-I, T3 and DIT on alkaline phosphatase activity in MC3T3-E1 cells: dose response to T3 (right) and lack of response to DIT (left).

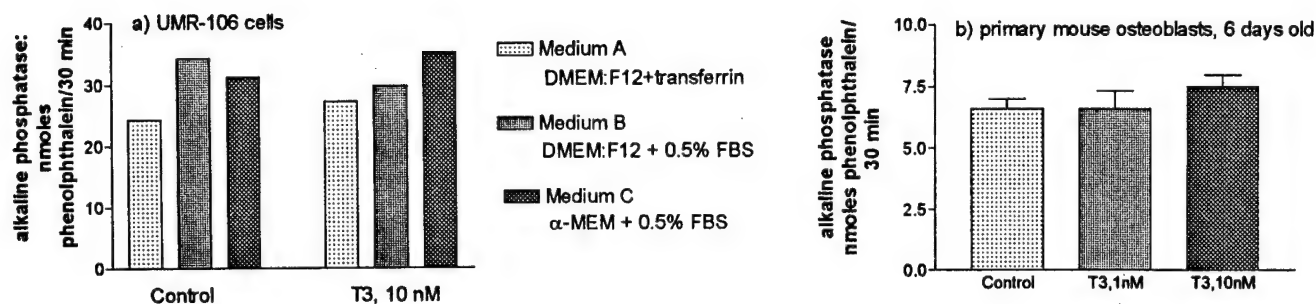


Figure B5. Effect of T3 on alkaline phosphatase activity in a) UMR-106 cells in 3 different media, and b) primary mouse osteoblasts.

Studies to determine the effects of antibody to the IGF-I receptor on T3 responses are shown in Figure B6. The effect of the antibody was surmounted when the T3 concentration was raised to 100 nM (Figure B6b). We believe that the antibody will be a more useful tool than IGFBP-3 for blocking IGF-I mediated effects. In the pilot study carried out to determine the effect of IGFBP-3 on the increase in alkaline phosphatase elicited by T3, we used 17.8 nM IGFBP-3, the maximum concentration we could achieve from the amount purchased. This failed to block the response to 10 nM T3. Although the experiment will be repeated with higher IGF-I and lower T3, the cost of commercial IGFBP-3 may make this approach less feasible than originally thought. Since decreasing the T3 concentration to 1 nM usually resulted in the loss of the response to T3, concentrations of 3-6 nM will be tested.

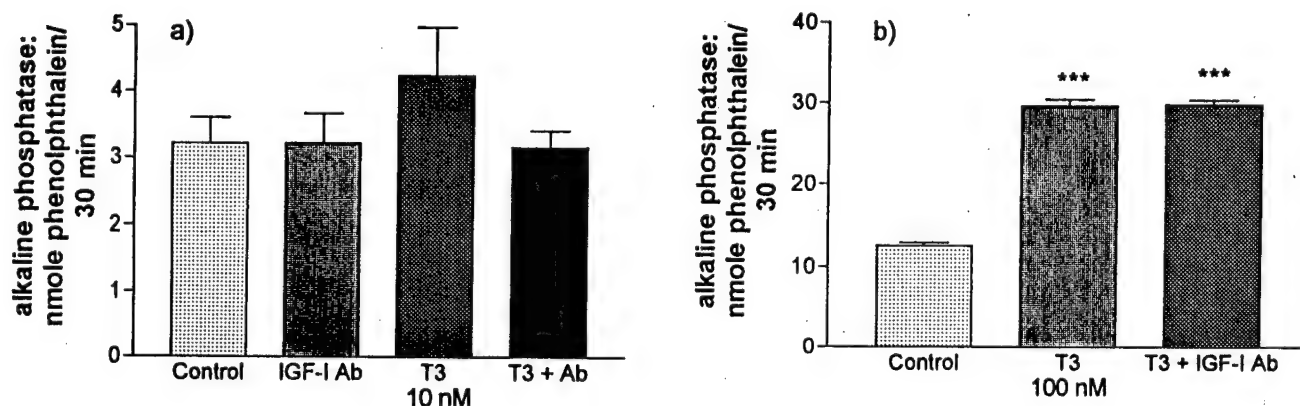


Figure B6. Effect of IGF-I receptor antibody on the effects of IGF-I on alkaline phosphatase activity in MC3T3-E1 cells.

Another approach that we used to determine the role of IGF-I in the effects of T3 was incubation with antisense oligonucleotides to the IGF-I receptor. Two phosphothiolated antisense oligonucleotides were used. The first was: 5'-GGACACCGCATCCAAGATGAGAGACCAGTCTATGGTGGAGAGG-3'. A non-phosphothiolated antisense oligonucleotide was also tested, although it was expected that this would not be sufficiently stable to have a persistent effect in the cultures. A phosphothiolated nonsense oligonucleotide, 5'-GATCGATCGATCGATCGATCGATCGATCGA-3' was used as the control for the 1st oligonucleotide. These sequences had been used previously in published literature (Wada et al. 1993). We have recently begun to test the effects of another sequence, a 15-mer found to antagonize the IGF-I receptor expression (Sergei Gryznov, personal communication, submitted for publication).

The first phosphothiolated antisense oligonucleotide antagonized the effects of IGF-I on alkaline phosphatase (*Figure B7*). The non-phosphothiolated antisense did not inhibit (*Figure B7*). The nonsense oligonucleotide was generally inhibitory, suggesting that this was not a good control. We will have a mismatch oligonucleotide prepared, which may be a better control. The effects of T3 on alkaline phosphatase were antagonized in some experiments (*Figure B8a*) but not in others (*Figure B8b*). The second antisense oligonucleotide has produced a dose-dependent decrease in the T3 response, with greater inhibition with 1 μ M than with 0.5 μ M (*Figure B9*). Currently we are carrying out experiments using extended preincubation with the antisense, and also are determining the optimal conditions for transfecting the cells with the oligonucleotide.

To demonstrate that the oligonucleotides decrease expression of the receptor we are carrying out binding studies and Western immunoblotting. In a binding experiment, (*Table B1*) we determined that the first phosphothiolated antisense oligonucleotide produced a decrease of about 20% in binding of IGF-I, from 38,110 sites/cell to 30,030 sites per cell. We are currently carrying out Western immunoblotting on cells treated with each of the oligonucleotides to determine their effects on the expression of the receptor. We have succeeded in demonstrating the IGF-I receptor in the MC3T3-E1 cells in Western immunoblots using an antibody from Santa Cruz, and are currently carrying out studies to examine the effects of the various antisense oligonucleotides on this expression, to optimize our treatment protocols.

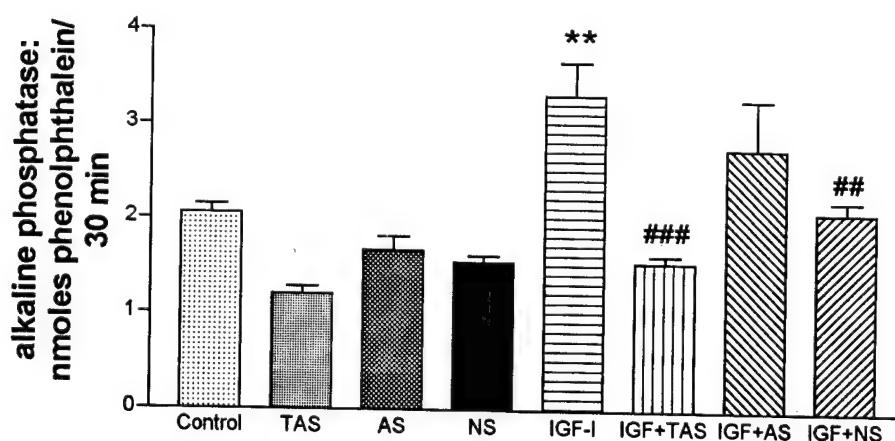


Figure B7. Effects of antisense and nonsense oligonucleotides on the alkaline phosphatase responses to IGF-I in MC3T3-E1 cells. (TAS: phosphothiolated; AS: nonphosphothiolated; NS: nonsense)

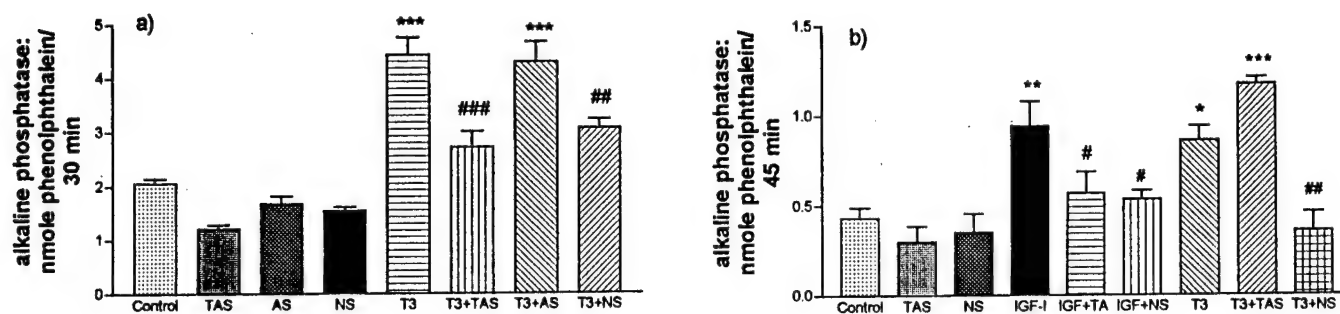


Figure B8. Effects of antisense and nonsense oligonucleotides on the alkaline phosphatase responses to T3 in MC3T3 cells;

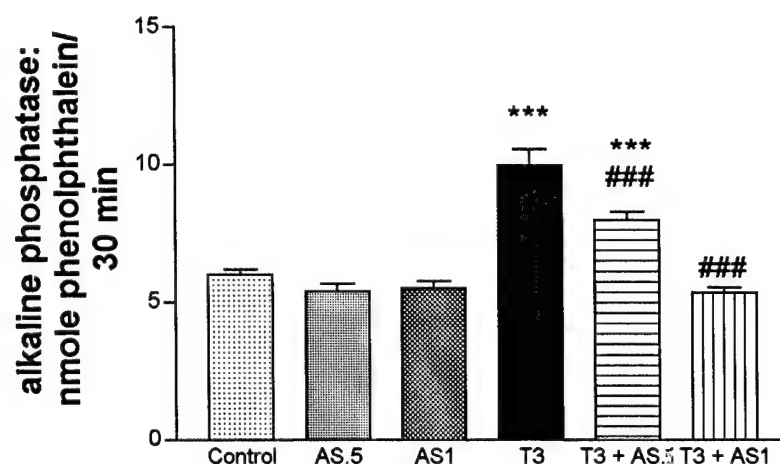


Figure B9. Dose-dependent inhibition by antisense oligonucleotide of the alkaline phosphatase response to T3 in MC3T3-E1 cells. The oligonucleotide was the 15-mer and the concentrations were 0.5 μ M and 1 μ M.

Table B1. IGF-I Binding parameters in MC3T3 cells treated with T3 and IGF-IR antisense

Treatment	% Specific Binding	IGF-I binding sites per cell
Control	15.5%	38,110
T3, 10 nM	14.1%	32,080
IGF-IR antisense, 0.5 μ M	13.0%	30,030
T3 + antisense	11.2%	27,980

T3 effects on IGF-I receptor binding: We also determined the effect of T3 on the binding of IGF-I to its receptors. T3 produced a dose-dependent down-regulation of the IGF-I receptor in MC3T3, UMR-106 and the osteoblastic osteosarcoma cell line ROS 17/2.8 after 72 hr incubation (Figure B10, Table B1).

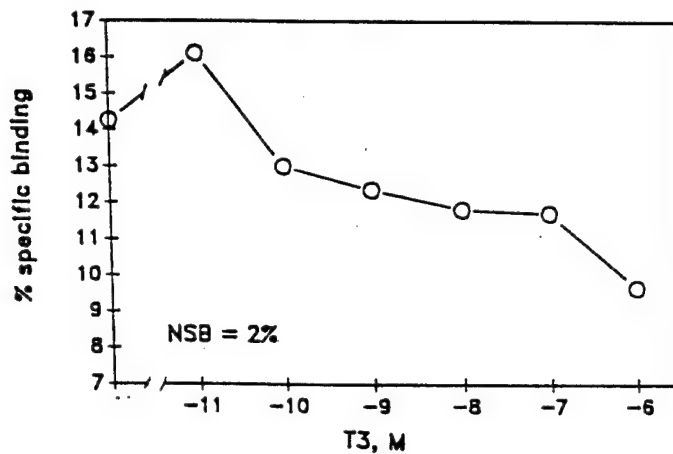


Figure B10. Down-regulation of IGF-I binding by 72h incubation of MC3T3-E1 cells with T3

The studies on alkaline phosphatase reveal that the MC3T3-E1 cell line is the optimal model of the three tested. The preliminary results obtained with antibody to the IGF-I receptor and with antisense oligonucleotides, especially the 15-mer, support a role for IGF-I in the actions of T3 to increase alkaline phosphatase activity in these cells. Further studies will be designed to optimize treatment protocols, determining the effects of pre-treatment in contrast with co-treatment. Also, Western blotting will allow us to determine the optimal conditions for inhibiting the expression of the receptor. The use of lower T3 (3 nM) may result in more consistent inhibition.

Effects of T3 on proline incorporation in calvarial cells: These studies were carried out initially in calvarial cells, because osteosarcoma cell lines generally have much lower rates of collagen synthesis. We carried out the study shown using a modified protocol, in which incorporation into total protein was used, rather than into collagenase digestible and non-collagen fractions, with the option of repeating the studies in the future using the collagenase digestion step to further discriminate between collagen and other proline-containing proteins. T3 stimulated proline incorporation in the calvarial cells and the 15-mer antisense oligonucleotide antagonized the effect of T3 (Figure B11). In future experiments, we will determine how this response is affected by IGF-I receptor antibody. We will also examine proline incorporation in the cell lines to determine how general the role of IGF-I might be in this effect of T3. The results further support the hypothesis that IGF-I plays a role in the anabolic effects of T3 on bone.

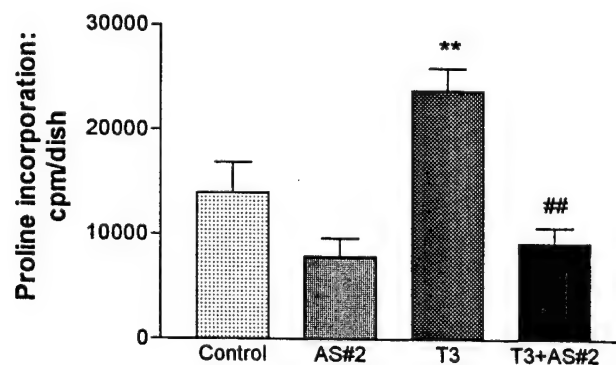


Figure B11. Effect of T3 and IGF-I antisense oligonucleotide on proline incorporation in neonatal mouse calvarial osteoblasts

Effects of T3 on osteocalcin: Osteocalcin is a bone-specific protein that is frequently used in addition to alkaline phosphatase to monitor osteoblastic activity. This method was new to our laboratory and it was necessary to initially develop the assay from components purchased from Biomedical Technologies. This was accomplished and very satisfactory binding curves have been obtained (*Figure B12*).

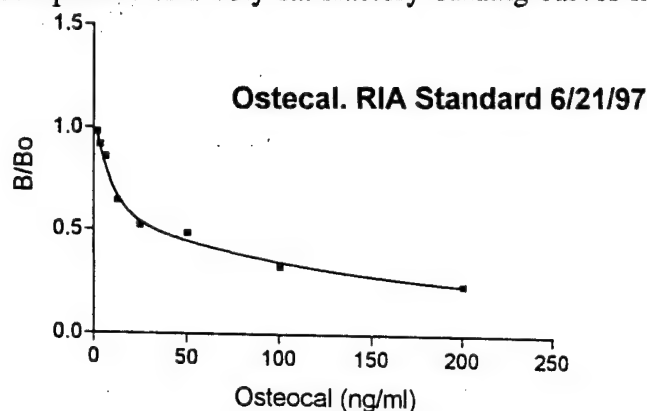


Figure B12. Osteocalcin radioimmunoassay standard curve

We carried out preliminary studies (N=2) to determine osteocalcin concentrations in media from MC3T3-E1 cells and calvarial cells to establish whether the assay could measure the concentrations in these tissues. UMR-106 cells do not make osteocalcin. Concentrations in non-stimulated MC3T3-E1 and calvarial cells were either just detectable or below the limits of the assay at the cell concentrations used. IGF-I, 1-10 nM, produced small stimulatory effects, and 10 nM T3 elicited a large stimulation in the MC3T3-E1 cells (*Figure B13a*). This stimulatory effect of T3 was not inhibited by 0.5 μ M of the 15-mer antisense (*Figure B13b*). In future experiments, higher concentrations of the antisense oligonucleotide and intermediate concentrations of T3 will be tested. In the calvarial cells, the antibody blocked the effect of IGF-I (*Figure B14a*), whereas the antisense oligonucleotide gave highly variable results (*Figure B14b*). Both the antibody to the IGF-I receptor and the antisense oligonucleotide inhibited the effect of T3 in the primary osteoblasts (*Figure B15*). These very limited preliminary studies will be repeated and extended with studies of dose-dependence and preincubation in further experiments. They likewise support the concept that IGF-I is a mediator of anabolic effects of T3.

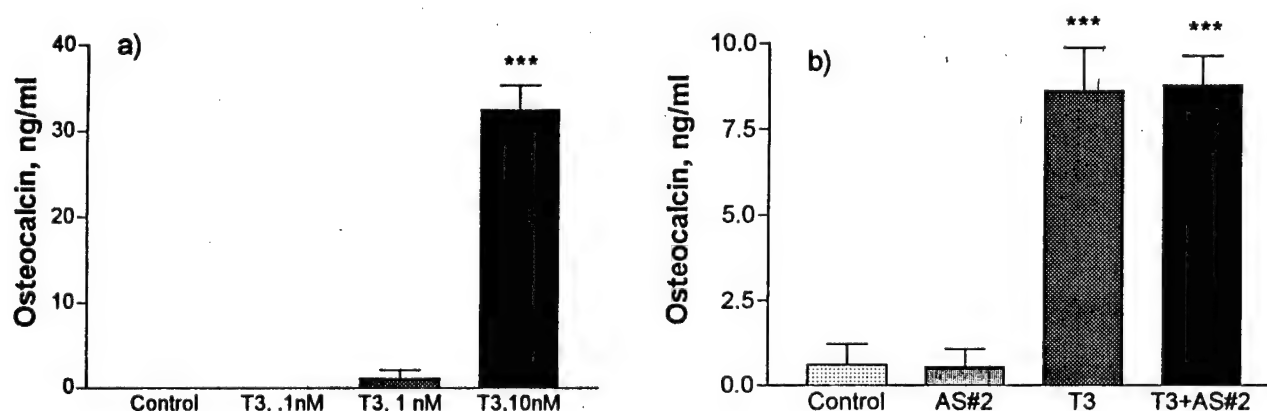


Figure B13. a) Dose-dependence of effect of T3 on osteocalcin in MC3T3-E1 cells; failure of antisense oligonucleotide to inhibit the effect.

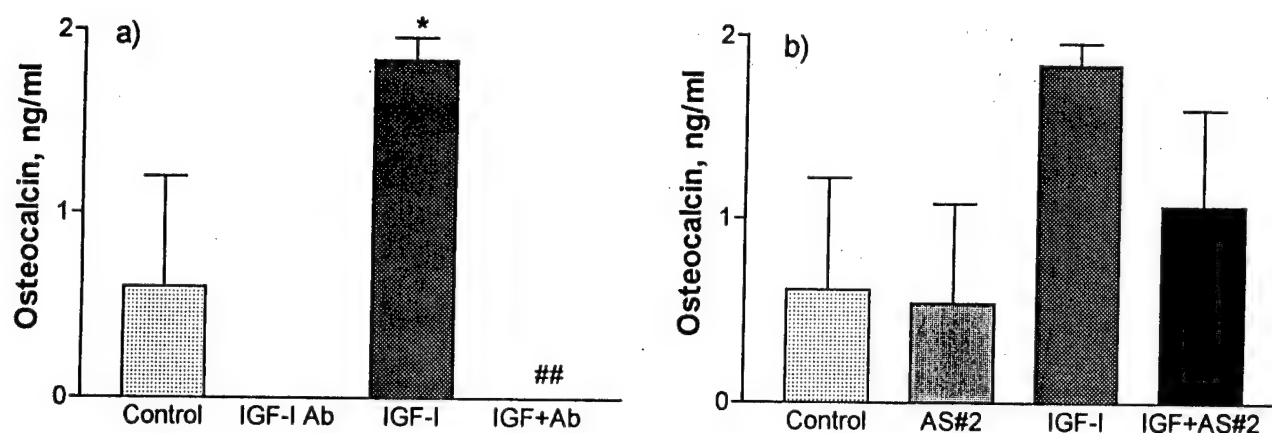


Figure B14. Effect of a) IGF-I receptor antibody and b) antisense oligonucleotide to the IGF-I receptor on the effects of IGF-I on osteocalcin in neonatal mouse calvarial cells.

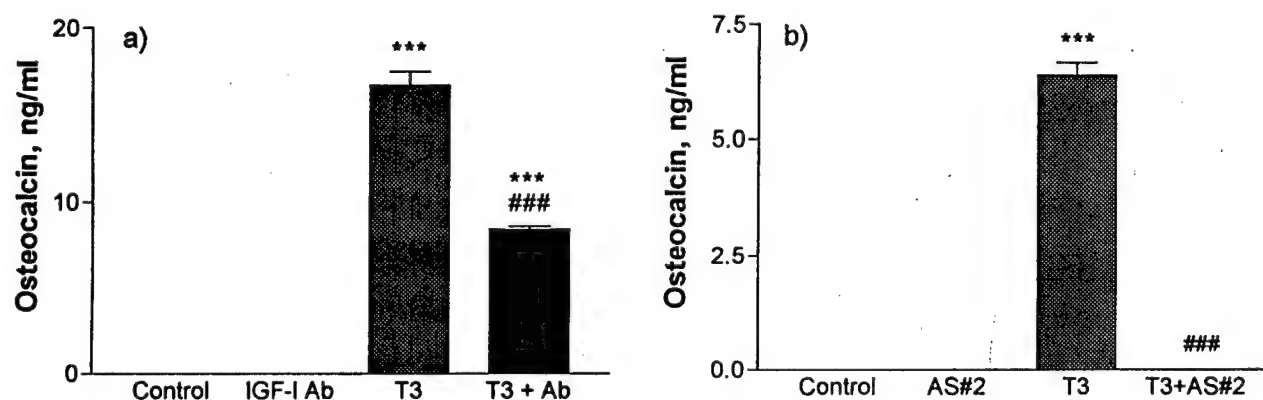


Figure B15. Effect of a) IGF-I receptor antibody and b) antisense oligonucleotide to the IGF-I receptor on the effects of T3 on osteocalcin in neonatal mouse calvarial cells.

Effect of IL-6 Ab and IL-6 receptor Ab on T3-stimulated bone resorption:

To investigate the role of IL-6 in the bone resorbing effects of T3, we used a model in which T3 has well-established effects that are not dependent upon prostaglandin production. The tools that we used to attempt to antagonize the T3 effect were an antibody to IL-6 and an antibody to the IL-6 receptor. The IL-6R Ab (Figure 16b), but not the IL-6 Ab (Figure 16a), decreased the effect of T3. Further studies will involve the use of higher concentrations of the antagonists. There may be a methodologic problem precluding increasing the concentrations of the IL-6 antibody. The antibody is reconstituted in phosphate buffered saline, and at the concentration of Ab required, the amount phosphate added to the cultures will inhibit resorption. We will attempt to carry out the study with antibody that has been constituted with a lower phosphate concentration. The preliminary findings on this aspect of the study support the concept that IL-6 generated by bone contributes to the bone-resorbing effects of T3.

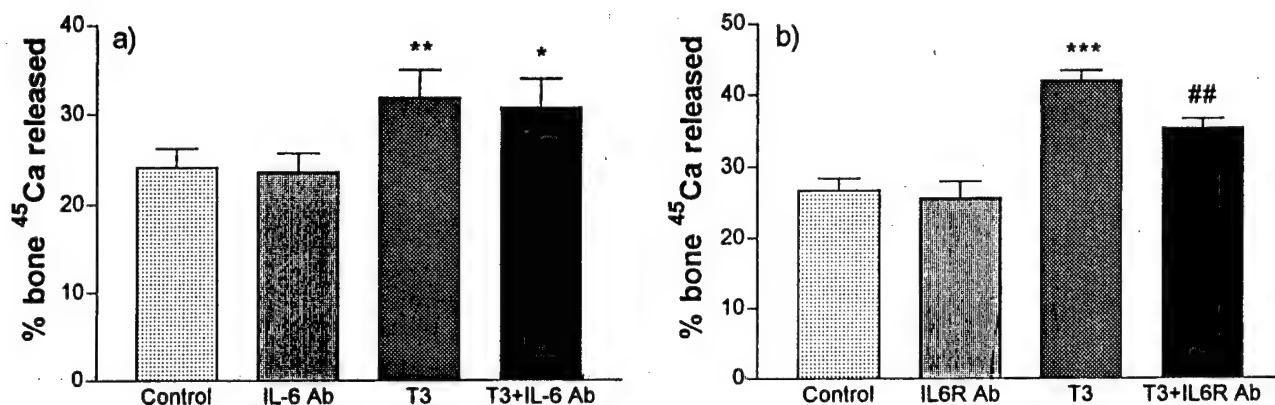


Figure B16. Effects of a) IL-6 Ab and b) IL-6 receptor Ab on T3 stimulated resorption in fetal rat limb bones

In summary, the work during the current year has established all of the methods proposed to be used for studying the anabolic effects of IGF-I and T3, thymidine incorporation, collagen synthesis, alkaline phosphatase, and osteocalcin production. The MC3T3-E1 cell line appear to be the best model for the studies, since each of the effects is measurable in this line and can be stimulated by IGF-I. All of the effect except thymidine incorporation are increased by T3 in the MC3T3-E1 cell line. It is not clear why T3 did not stimulate thymidine incorporation, although in other studies T3 has been found to show different effect on cell proliferation. The conditions of serum starvation used to quiesce the cells may have removed an additional factor that is necessary for T3 to stimulate cell proliferation.

Both IGF-I and T3 stimulated the parameters that were indicative of osteoblast differentiation in the anabolic direction, including alkaline phosphatase, osteocalcin and collagen synthesis. For the investigations of the role of IGF-I in the anabolic effects of T3, we initiated feasibility studies during this year using three approaches: the use of excess IGFBP-3, the use of antibodies to the IGF-I receptor (which had not been proposed in the original application, as the anti rat/mouse receptor antibody was not available at that time) and the use of antisense oligonucleotides to the receptor. Our initial attempt to block the effects with IGFBP-3, were unsuccessful. The obvious follow-up experiment is to decrease the concentration of T3 and to increase the concentration of IGFBP-3. However, we decided to test the other approaches before undertaking any further investment in this material. An antibody to the IGF-I receptor, which was not available at the time our proposal was written, has given some interesting results, inhibiting effects of T3 on each of the parameters tested. A third approach, which was in the original proposal was the use of antisense oligonucleotides. We tested two antisense sequences, that paired with different regions of the IGF-R message. Again, our preliminary experiments have indicated that such oligonucleotides can inhibit the effect of T3. A number of further experiments are required to validate the mechanism of the effects of the oligonucleotides, especially since a nonsense oligonucleotide was also inhibitory. The most useful approach will be Western immunoblotting to assess the extent to which the expression of the target protein has been decreased, and the specificity of the response. If the specificity of the effect is validated, we will introduce modifications in the protocols, to attempt to improve the

responses. In the preliminary experiments, we used a co-incubation protocol. Responses were sometimes inconsistent, as shown by *Figures 8*. In future experiments we will test the effect of pre-incubation, and will also determine responses after transfection of cells with the oligonucleotides, methods that we are just beginning to test with Lipofectin®.

Studies on the role of IL-6 in the resorptive response to T3 have been more limited than the studies of the anabolic effects. However, we have found that antibody to IL-6R can decrease the effect of T3 to stimulate bone resorption in the fetal rat limb bones. This result supports the hypothesis that IL-6 plays a role in the bone loss elicited by T3. The inhibitory effect was small, suggesting that IL-6 may contribute to the response, but not play a major role. It will be necessary to develop approaches to be able to increase the amount of antibody, without increasing the phosphate concentration to unacceptable levels with respect to bone resorption. It may be possible to identify a better model to study the role of the IL-6 response in T3-stimulated resorption. A recent abstract (Tomita, et al 1997) showed that adult mouse vertebrae undergo IL-6 stimulated resorption in culture. We have previously used a neonatal vertebral model for organ culture studies of resorption (Stewart and Stern, 1987) which might prove to be a more sensitive model for these studies. Although this could be an interesting approach, we are aware that studies have shown that in 8-week old rats, T3 stimulation of marker gene expression in osteoblasts from vertebral bone did not elicit the responses seen in femoral bone (Suwanwalaikorn, et al. 1997).

Recommendations in Relation to the Statement of Work

Sections IA and IIA

Our experimental approach of using osteosarcoma cell lines to examine T3 effects on IL-6 and IGF-I gene expression has not fully supported the initial expectation we reached with our preliminary data. We have examined the issue very closely, studying nine different cell lines in over 60 experiments during the first year of the project. There have been three types of disappointing results; a) some cell lines fail to show any synergy between T3 and IL-1 in producing IL-6, b) some cells, notably SaoS-2 and MG-63 have shown variable amounts of T3/IL-1 synergy ranging between a two fold effect in mRNA to a 7 fold effect in secreted IL-6, c) The response of the cell lines have not been stable, with the physiology tending to disappear with even moderate passage (3-5 passages) of the cells and sudden, unpredictable, loss of the expected response.

There are several alternatives to working around this problem, which we propose as necessary changes in our initial strategy outlined in the statement of work. The comments below refer equally to both the IL-6 and the IGF-I arms of the study. The techniques and experience gained from the first year of study will make the implementation of these new strategies a straight forward achievable goal.

First, the large synergistic T3/IL-1 response reported by Dr. Stern (Tarjan, 1995) was observed in mouse calvaria which is immature bone, of rodent origin, and contains a mixture of osteoblasts and osteoclasts. We will therefore examine in detail the MC3T3-E1 cell line which is an immature mouse osteoblast cell line. This will require development of new primer pairs for IL-6 and IGF-I RT-PCR and new antibodies for IL-6 and IGF-I ELISA, but the technical issues should be easily resolved.

Second, since we are fundamentally interested in the human biology, we have also begun to plan similar experiments using cultures of primary human bone cells. The exact approach has not yet been detailed, and no experiments with human tissue have yet been proposed to our Institutional Review Board. When available we will submit to your office for evaluation any proposals to work with donated human

tissue as required by our institutional regulations. We are developing a practical strategy for obtaining the human bone cells in a routine fashion in collaboration with several orthopedic surgeons. Material obtained from human greater trochanter during total hip replacement is likely to be the main source of human material when we proceed with this line of investigations. Methods for obtaining and maintaining cell cultures from human material have been described (Gundle, 1995). These mixed cultures of osteoblasts and osteoclasts may be a far superior model than the osteosarcoma cell line in examining the IGF-I and IL-6 responses of the cells to T3 and IL-1, but their limited duration in culture and reduced reproducibility will significantly limit our options in terms of detailed molecular biology experiments.

Third, we will directly examine several key issues concerning thyroid hormone regulation of the IL-6 and IGF-I genes, by proceeding directly to analysis of the promoter constructs we have available without having first been able to demonstrate the desired physiology from the native cellular genes. Because of the ease with which the stoichiometry of interacting proteins and genes can be manipulated in transient gene expression assays, it is often possible to demonstrate molecular interactions which might otherwise be subtle among the forces controlling native gene expression. A major obstacle to this third approach is developing an efficient transfection technique for osteosarcoma cells. Several new commercial products for such applications have recently been developed and we will test these (FuGene, Tfx reagent, etc.). However, if transient gene expression remains inefficient, two additional options will be explored. First stable, transfected, cell lines will be created by electroporation and antibiotic selection with the IL-6 and IGF-I promoter plasmids and a co-transfected gentamycin resistance plasmid. Although time consuming, this approach is a standard one for overcoming issues of low transfection efficiency.

A second option we are exploring is to examine the molecular interactions thought to be responsible for the T3/IGF-I and T3/IL-1 and IL-6 physiology directly in a cell line which allows for easier transfection and more abundant expression of the promoter in question (Cos-1, α T3, Hela, etc.). Both IGF-I and IL-1 are widely expressed in human tissues, and it is possible that insight into the bone physiology may be obtained in such a heterologous system. Some specific issues to be examined with this later approach include the functional significance of two apparent TR binding site in the proximal IGF-I promoter (-400 to -386 and -341 to -323), examination of the functional interaction of the TR and the newly discovered estrogen receptor beta (ER β) isoform in controlling IL-6 gene expression. The ER has been described as a major physiologic suppressor of the IL-6 gene, responsible by its de-repression for the major bone loss occurring after menopause. One hypothesis for T3 induced bone damage stated in our initial proposal is the TR, activated by T3 competes with and is able to block the tonic repression of the IL-6 gene for which the ER is responsible. The discovery of a new isoform of this well studied receptor family has generated a great deal of excitement and hypothesis generation (Kuiper, 1996). Of note is the observation that the ER β is abundantly expressed in bone, in excess of the amount of ER α and maybe responsible for the majority of effect of estrogen on bone. We have a separate project in the lab examining the biology of the ER β , so an efficiency of resources will be achieved in these studies.

Sections IB and IIB: Our studies during the first year of the grant have result in considerable progress towards the goals of Specific Aim IB in that we have established the proposed assays, determined the optimal model, and begun to study the effects of interventions. We have taken advantage of a valuable reagent that was not available at the time we submitted the proposal, the antibody to the rat/mouse IGF-IR, and have used this rather than excess IGFBP-3, as it appears to be a more potent antagonist, and one which more specifically addresses the questions asked. We have made smaller advances towards the goals of Specific Aim IIB, although the findings that antibody to the IL-6 receptor inhibited the response to T3 is certainly noteworthy. During the next year, with reference to Specific Aim IB, we will be carrying out further experiments to test the

effects of IGFBP-3, antibody to the IGF-I receptor and the antisense oligonucleotides on the anabolic effects of T3. Particularly, we will be validating the use of the antisense oligonucleotides by testing their specificity by Western immunoblotting. We will also be modifying the conditions for studying thymidine incorporation to attempt to obtain a T3 effect and to then study its mechanism. With respect to Specific Aim IIB, we will carry out further studies on the effects of the IL-6 and IL-6 receptor antibodies, modifying the solvent to allow us to increase the concentrations of the antibodies in the medium. We will also determine whether vertebral bones might be a more sensitive system for studying the role of IL-6 in the resorptive response to T3. We will also establish the effects of T3 and intervention with the IL-6 system on other parameters of the resorptive response, the enzymes collagenase and cathepsin B.

CONCLUSIONS

The model systems for investigations of T3 control of IGF-I and IL-6 gene regulation by T3 have been proposed in sections IA and IIA have been elaborated. No single ideal tissue culture model system has been found for study of both IGF-I and IL-6. A great deal of time and effort went into comparing different paradigms of cell type, length of culture, method, dose and time-course of T3 treatment. Greater success has been obtained with the IL-6 arm of the studies in general. The IGF-I studies have revealed very low to non-detectable levels of IGF-I expression in human osteosarcoma cell lines, confirming a similar opinion stated in the literature, but debated and contradicted by other work. IGF-I studies will necessarily be limited to rodent cell lines (MC3T3--E1) in ongoing and future studies.

A robust RT-PCR assay for low level gene expression in osteosarcoma cell lines is established and has been further improved. We now use a commercial oligonucleotide product, 18S Competicon (Ambion) to quantitate 18S RNA as the internal control for measurement of experimental genes. This technical improvement overcomes problems with both biological controls and competitive controls, detailed in the grant application. We have used the RT-PCR assay to document: 1) a modest 1.5 to 2.0 fold T3 augmentation of IL-1 induced IL-6 mRNA levels in human osteosarcoma cells; 2) that T3 treatment reduces the level of IL-1 receptor mRNA, opposite the anticipated effect and opposite the effect previously observed with Vit. D which also potentiates IL-1 induced IL-6 secretion. This suggests that T3 and Vit D augment IL-6 production through different mechanisms

The experiments in Part IA of the project have progressed less than the IL-6 arm of the study, primarily because of reduced time and effort on this arm of the project because of the need for continued attention to the problems described above with the IL-6 studies in osteosarcoma cells. In addition we have had technical difficulties with IGF-I measurements for three major reasons: 1) very low to unmeasurable production of IGF-I in human cell lines, 2) an unusual biphasic and large competitive interaction between cyclophilin and IGF-I cDNA amplification products. We have been unable to explain the basis for this unusual effect. The Competicon 18S primer techniques has eliminated this unusual technical problem.

With respect to Specific Aims IB and IIB, the data obtained in the first year of the studies demonstrate the feasibility of the approaches proposed, with the possible exception of the use of excess IGFBP-3 to block responses. The preliminary results with antibodies and antisense oligonucleotides have provided evidence that would support the hypotheses that the T3-stimulated increase in IGF-I and the T3 enhancement of the IL-1-stimulated IL-6 secretion contribute, respectively, to the anabolic and bone resorbing effects of the hormone. Future studies will repeat, refine and extend these studies.

REFERENCES.

- Allain, T. J.; Chambers, T. J.; Flanagan, A. M.; McGregor, A. M. *J Endocrinol* **1992**, *133*, 327-331.
- Baran, D. T.; Braverman, L. E. *J Clin Endocrinol Metab* **1991**, *72*, 1182-1183.
- Black, K.; Garrett, I. R.; Mundy, G. R. *Endocrinology* **1991**, *128*, 2657-2659.
- Bordier, P.; Miravet, L.; Matrajt, H.; Hioco, D.; Ryckewaert, A. *Proc Roy Soc Med* **1967**, *60*, 26.
- Britto, J. M.; Fenton, A. J.; Holloway, W. R.; Nicholson, G. C. *Endocrinology* **1994**, *134*, 169-176.
- Canalis, E. *J. Clin. Invest.* **1980**, *66*, 709-719. Centrella, M.; McCarthy, T. L.; Canalis, E. *Endocrinology* **1990**, *126*, 39-44.
- Cooper, D. S.; Black, D. M.; Rubin, S. M. *Ann. Intl. Med.* **1979**, *90*, 164-168.
- Diamond, T.; Nery, L.; Hales, I. *J Clin Endocrinol Metab* **1991**, *72*, 1184-1188.
- Egrise, D.; Martin, D.; Neve, P.; Verhas, M.; Schoutens, A. *Bone Miner* **1990**, *11*, 273-283.
- Ernst, M.; Froesch, E. R. *FEBS Lett.* **1987**, *220*, 163-166.
- Franklyn, J. A.; Sheppard, M. C. *Brit. Med. J.* **1990**, *300*, 693-694.
- Gundle R. Beresford J.N. *Calcif Tiss Internat* **1995**, s8-s10.
- Guo, C.Y.; Weetman, A.P.; Eastell, R. *Clin Endocrnol* **1997** *46*, 301-307
- Harvey, R. D.; McHardy, K. C.; Reid, I. W.; Paterson, F.; Bewsher, P. D. *J Clin. Endoc. Metab.* **1991**, *72*, 1189-1194.
- Hock, J.; Centrella, M.; Canalis, E. *J Bone Min Res* **1986**, *1*, 67.
- Hoffmann, O.; Klaushofer, K.; Koller, K.; Peterlik, M.; Mavreas, R.; Stern, P. *Prostaglandins* **1986**, *31*, 601-608.
- Hoyland, J. A.; Freemont, A. J.; Sharpe, P. T. *J Bone Miner Res* **1994**, *9*, 75-80.
- Kasono, K.; Sato, K.; Han, D. C.; Fujii, Y.; Tsushima, T.; Shizume, K. *Bone Min.* **1988**, *4*, 355-363.
- Kassem, M.; Mosekilde, L.; Eriksen, E. F. *Biochem Mol Biol Int* **1993**, *30*, 779-788.
- Kawaguchi, H.; Pilbeam, C. C.; Raisz, L. G. *Endocrinology* **1994**, *135*, 971-976.
- Kawaguchi, H.; Pilbeam, C. C.; Woodiel, F. N.; Raisz, L. G. *J Bone Miner Res* **1994**, *9*, 247-253.
- Klaushofer, K.; Varga, F.; Glantschnig, H.; Fratzi-Zelamn, N.; Czerwenka, E.; Leis, H. J.; Koller, K.; Peterlik, M. *J. Nutrit.* **1995**, *125*, 1996s-2003s.
- Krieger, N. S.; Stappenbeck, T. S.; Stern, P. H. *J. Bone Min. Res.* **1988**, *3*, 473-478.
- Kuiper G.; Enmark E.; Peltö-Huikko M; Nilsson S.; Gustafsson J.A. **1996** *Proc Natl Acad Sci USA*, *93*, 5925-5930
- Kurihara, N.; Bertolini, D.; Suda, T.; Akiyama, Y.; Roodman, G. D. *J Immunol* **1990**, *144*, 4226-4230.
- Lacey, D.L.; Grosso, L.E.; Moser S.A.; Erdmann J.; Tan H.R.; Pacifici R. Villareal D.T. *J Clin Invest*, **1993**, *91*, 1731-1742
- Lakatos, P.; Caplice, M. D.; Khanna, V.; Stern, P. H. *J Bone Miner Res* **1993**, *8*, 1475-1481.
- Lakatos, P.; Foldes, J.; Kiss, L.; Tarjan, G.; Stern, P. H. *Bone* **1995**, *16(S1)*, 183S.
- Lakatos, P.; Kiss, L.; Tatrai, A.; Foldes, J.; Tarjan, G.; Stern, P. H. *J. Clin. Endocrinol Metab.* **1997**, *82*, 78-81.
- LeBron, B. A.; Pekary, A. E.; Mirell, C.; Hahn, T. J.; Hershman, J. M. *J. Bone Min. Res.* **1989**, *4*, 173-178.
- Lee, M. S.; Kim, S. Y.; Lee, M. C.; Cho, B. Y.; Lee, H. K.; Koh, C. S.; Min, H. K. *J. Clin. Endoc. Metab.* **1990**, *70*, 766-770.
- Lowe W.L. Teasdale R.M. *Biochem Biophys Res Comm* **1992**, *7*, 797-805
- Manolagas, S. C.; Jilka, R. L. *New Eng. J. Med.* **1995**, *332*, 305-311.
- McCarthy, T. L.; Centrella, M.; Canalis, E. *Endocrinology* **1989**, *124*, 1247-53.
- Mosekilde, L.; Eriksen, E. F.; Charles, P. *Endoc Metab Clin* **1990**, *19*, 35-63.

- Mosekilde, L.; Melsen, F. *Acta Med. Scand.* **1978**, *204*, 97-102.
- Mundy, G. R.; Shapiro, J. L.; Bandelin, J. G.; Canalis, E. M.; Raisz, L. G. *J. Clin. Invest.* **1979**, *58*, 529-534.
- Passeri, M.; Girasole, G.; Guiliani, N.; Pedrazzoni, M.; Roti, E.; Salvi, M.; Minelli, R.; Gatti, C.; Campanini, C. *J. Bone Min. Res.* **1995**, *10*, S143.
- Pirskanen, A.; Jaaskelainen, T.; Maenpaa, P. H. *Eur J Biochem* **1993**, *218*, 883-891.
- Reddy, S. V.; Takahashi, S.; Dallas, M.; Williams, R. E.; Neckers, L.; Roodman, G. D. *J Bone Miner Res* **1994**, *9*, 753-757.
- Refetoff, S.; Weiss, R. E.; Usala, S. J. *Endoc. Rev.* **1993**, *14*, 348-399.
- Riggs, B. L.; Melton, L. J. *N. Engl. J. Med.* **1986**, *314*, 1676-1686.
- Rizzoli, R.; Poser, J.; Burgi, U. *Metabolism* **1986**, *35*, 71-74.
- Roodman, G. D.; Kurihara, N.; Ohsaki, Y.; Kukita, A.; Hosking, D.; Demulder, A.; Smith, J. F.; Singer, F. R. *J Clin Invest* **1992**, *89*, 46-52.
- Rose J.K.; Buonocore L.; Whitt M.A.; *BioTechniques* **1991**, *10*, 520-525
- Samuels H.H.; Stanley F.; Casanova J.; *Endocrinology* **1979**, *105*, 80-85.
- Sanders, J.L.; Stern, P.H.; *J Bone Miner Res* **1996**, *11*, 1862-1867.
- Sato, K.; Han, D. C.; Fujii, Y.; Tsushima, T.; Shizume, K. *Endocrinology* **1987**, *120*, 1873-1881.
- Schlossman, M.; Brown, M.; Shapiro, E.; Dziak, R. *Calcif Tiss Int* **1982**, *34*, 190-196.
- Schmid, C.; Schlapfer, I.; Futo, E.; Waldvogel, M.; Schwander, J.; Zapf, J.; Froesch, E. R. *Acta Endocrinol* **1992**, *126*, 467-73.
- Stern B.; Yang M.X.; *Molec Cell Biol* **1995**, *15*, 4971-4979
- Stewart, P.J.; Stern, P.H. *Calc Tiss Intl* **1987** *40*:21-26.
- Stoll, G. M.; Harris, S.; Sokoll, L. J.; Dawson-Hughes, B. *Ann. Intern. Med.* **1990**, *113*, 265-269.
- Suwanwalaikorn, S.; Van Auken, M.; Kang, M.I., Alex, S., Braverman, L.E., Baran, D.T. *Amer J Physiol* **1997**, *272*, E212-E217
- Tarjan G.; Stern P.H. *J Bone Min Res.* **1995**, *10*, 1321-1326
- Tarjan, G.; Stern, P. H. *J. Bone Min. Res.* **1995**, *10*, 1321-1326.
- Tomita, T.; Miyuara, C.; Yamauchi, H.; Inoue, T.; Morita, R.; Ozawa, H.; Suda, T.; Orimo, H. *J Bone Miner Res* **1997**, *12*, S191.
- Varga, F.; Rumpler, M.; Klaushofer, K. *FEBS Lett* **1994**, *345*, 67-70.
- Wada, J.; Liu, Z.Z.; Alvares, K.; Kumar, A.; Wallner, E.; Kanwar, Y.S. *Proc Natl Acad Sci* **1993**, *90*, 10360-10364.
- Williams, G. R.; Bland, R.; Sheppard, M. C. *Endocrinology* **1994**, *135*, 2375-2385.